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Mutational Analysis of the Human *MAOA* Gene

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The monoamine oxidases (MAO-A and MAO-B) are the enzymes primarily responsible for the degradation of amine neurotransmitters, such as dopamine, norepinephrine, and serotonin. Wide variations in activity of these isozymes have been reported in control humans. The *MAOA* and *MAOB* genes are located next to each other in the p11.3–11.4 region of the human X chromosome. Our recent documentation of an MAO-A-deficiency state, apparently associated with impulsive aggressive behavior in males, has focused attention on genetic variations in the *MAOA* gene. In the present study, variations in the coding sequence of the *MAOA* gene were evaluated by RT-PCR, SSCP, and sequencing of mRNA or genomic DNA in 40 control males with >100-fold variations in MAO-A activity, as measured in cultured skin fibroblasts. Remarkable conservation of the coding sequence was found, with only 5 polymorphisms observed. All but one of these were in the third codon position and thus did not alter the deduced amino acid sequence. The one amino acid alteration observed, lys→arg, was neutral and should not affect the structure of the protein. This study demonstrates high conservation of coding sequence in the human *MAOA* gene in control males, and provides primer sets which can be used to search genomic DNA for mutations in this gene in males with neuropsychiatric conditions.

KEY WORDS: MAO, polymorphisms, human

INTRODUCTION

The monoamine oxidases, MAO-A and MAO-B, are isozymes which are differentially expressed during development and in different tissues [reviewed in Breakefield et al., 1994; Shih, 1991; Kwan et al., 1992], and which have distinctive 5′ regulatory elements [Zhu et al., 1992; Denney et al., 1994]. They have a critical role in the degradation of a large number of biogenic amines, including neurotransmitters, e.g., norepinephrine, serotonin, dopamine, and histamine, and dietary amines that can act as "false" transmitters [reviewed in Weyler et al., 1990; Reiderer et al., 1989]. Variations in monoamine oxidase genes may underlie differences in physiology in humans that can manifest as mood or behavioral disorders, mental retardation, sensitivity to diet or drugs, cardiovascular problems, or other neurologic symptoms [reviewed in Hsu et al., 1989; Donnelly et al., 1979; Cohen, 1987; Brunner et al., 1993a,b].

Normal variations in MAO-A activity, as measured in cultured skin fibroblasts, and MAO-B activity, as measured in platelets, appear to be genetically determined in humans [Murphy et al., 1976; Rice et al., 1984; Costa et al., 1980; Breakefield et al., 1980]. In the case of MAO-A, this genetic variation appears to be mediated predominantly by the *MAOA* locus, based on allele association studies [Hotamisligil and Breakefield, 1991], whereas for MAO-B, the role of the structural gene is not clear [Girman et al., 1992]. The *MAOA* and *MAOB* genes lie next to each other in the p11.3–11.4 region of the X chromosome [Chen et al., 1992b], with the *MAOB* gene more centromeric and next to the Norrie disease gene [Sims et al., 1992; Chen et al., 1992a]. Norrie disease is a syndrome of congenital blindness, sometimes accompanied by mental retardation and progressive hearing loss [Warburg, 1974]. Submicroscopic chromosomal deletions, including the *MAOA*, *MAOB*, and Norrie disease genes, result in a contiguous gene syndrome, including congenital blindness, severe mental retardation, and seizures [Sims et al., 1989; Lan et al., 1989; Donnai et al., 1988; Collins et al., 1992; Gal et al., 1986; Zhu et al., 1989]. Selective disruption of the *MAOA* gene, caused by a single base pair substitution which introduces a stop codon into the amino acid sequence, underlies a syndrome of mild mental retardation and disrupted regulation of impulse control in a family in the Netherlands [Brunner et al., 1993a,b].

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In this study we sought to elucidate mutations in the coding region of the *MAOA* gene, which might underlie variations in enzyme activity measured in control human skin fibroblasts, based on known cDNA sequence [Hsu et al., 1988; Bach et al., 1988] and gene structure [Chen et al., 1991; Grimsby et al., 1991]. Analysis was carried out using a reverse transcriptase (RT)-polymerase chain reaction (PCR) procedure and direct sequencing of cDNA from fibroblast strains from 10 males varying over 100-fold in levels of MAO activity. Furthermore, flanking intronic sequences were extended to allow PCR amplification of all 15 exons of the *MAOA* gene. PCR fragments were analyzed for single-strand conformational polymorphisms [SSCPs; Orita et al., 1989], using genomic DNA from 40 males, and fragments with altered migration were sequenced.

MATERIALS AND METHODS

Reverse Transcriptase (RT)-PCR

For the synthesis of first-strand cDNA, total cellular RNA was extracted from cultured fibroblasts using the guanidinium thiocyanate method [Chirgwin et al., 1979]. After phenol/chloroform extractions, total RNA was ethanol-precipitated, and the pellets were air-dried and dissolved in H_2O. First-strand cDNA synthesis was performed on 30 μg total cellular RNA using oligodeoxythymidine (oligo dT) (Pharmacia, Piscataway, NJ) and Moloney murine leukemia virus reverse transcriptase, under the conditions recommended by the manufacturer (BRL). Following phenol-chloroform extractions, single-stranded cDNA was ethanol-precipitated and then dissolved in 20 μl 10 mM Tris-1 mM EDTA buffer, pH 7.4. One microliter was subjected to 30 cycles of amplification using a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) [Mullis and Faloona, 1987] and Taq polymerase (Boehringer-Mannheim). Primers (100 ng) corresponding to human MAOA cDNA nucleotides 84–102 (5′-AAGAGAAGGCGAGTATCGC-3′) and to nucleotides 1889–1908 (5′-GATCACAAGGCTTTATTCTA-3′) [Hsu et al., 1988] permitted double-stranded amplification of the entire coding sequence. Amplification conditions consisted of an initial denaturing step at 94°C for 2 min; 30 cycles of denaturing at 94°C for 1 min, and annealing at 50°C for 2 min; polymerase extension at 72°C for 3 min; and a final extension for 10 min at 72°C using 1.25 units of Taq polymerase (in buffer supplied by the manufacturer) in a 50-μl reaction. PCR products were resolved by electrophoresis in 0.8% agarose gels to check for purity.

The double-stranded PCR product was used as a template to generate single-stranded DNA by priming multiple rounds of DNA synthesis with only one of the oligonucleotides previously used in the double-stranded reaction [Gibbs et al., 1989]. Conditions for the single-strand generation were identical to those for PCR amplification of cDNA, as described above. After 30 cycles, the product was ethanol-precipitated in the presence of ammonium acetate, and resuspended in water for subsequent sequence analysis.

Detection of Single-Strand Conformation Polymorphisms (SSCP)

The polymerase chain reaction was used to amplify each exon from approximately 100 ng genomic DNA obtained from cultured fibroblasts. PCR reactions contained 1 × amplification buffer (Boehringer-Mannheim), 200 μM dATP, dCTP, dGTP, and dTTP, 100 ng of each primer, and 1.25 units of Taq polymerase, as modified from Orita et al. [1989]. After initial heating at 94°C for 2 min, the DNA was subjected to 30 cycles of amplification of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a single extension step of 72°C for 10 min. One microliter of the reaction product was amplified a second time using the same primers in the presence of [alpha ^{32}P] dGTP (3,000 Ci/mmol; Amersham/Searle, Arlington Heights, IL) and 50 μM dGTP. An aliquot of the ^{32}P-labelled reaction was diluted 1 : 50 in 10 mM EDTA/0.1% SDS. Three microliters of the diluted sample were added to 3 microliters of a solution containing xylene cyanol, Bromophenol blue, and formamide (U.S. Biochemicals, Cleveland, OH), and boiled for 5 min. The samples were cooled on ice, and loaded immediately onto a nondenaturing 6% (or 8% for exons 14 and 15) acrylamide/5% glycerol gel. SSCP gels were run at room temperature overnight at 5–10 watts. Gels were dried under vacuum and placed on film with an intensifying screen overnight at −80°C.

DNA Sequencing

Double-stranded DNA, which was used as sequencing template to obtain the intronic sequence, was alkali-denatured prior to primer annealing (USB Sequenase protocol). The DNA was then incubated with 100 ng of primer with Sequenase buffer U.S. Biochemicals at 37°C for 30 min, then sequenced according to manufacturer's instructions. To obtain the exonic sequence, which was used to check for mutations, ssDNA was first produced via asymmetric PCR. The conditions were the same as for double-stranded PCR, except that only one primer was added to the reaction mix, and previously amplified dsDNA was used as template. This ssDNA was precipitated twice in ethanol, and then resuspended in 10 μl H_2O. To this was added 100 ng of appropriate primer and 2 μl of Sequenase buffer U.S. Biochemicals. This was heated to 95°C for 3 min, and then 65°C for 5 min. The rest of the sequencing procedure was the same as according to manufacturer's instructions. The termination reactions, performed at 37°C for 5 min, were stopped by adding 4 μl stop buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, and 0.05% xylene cyanol), and heated to 95°C for 3 min. A portion of each termination reaction was loaded on a 0.4-mm-thick 6% polyacrylamide/8 mM urea gel. Electrophoresis was performed at 70 W for 1.5–4 hr, followed by gel drying and exposure to Kodak X-omat film overnight at room temperature.

RESULTS

Sequencing of the complete coding region of MAO-A cDNAs was carried out for 10 fibroblast strains from 7 control males and 3 Lesch-Nyhan males, who mani-

fested MAO-A activities over a 100-fold range (as measured in previous studies [Edelstein et al., 1978; Costa et al., 1980]; Table I). These were compared with previously published sequences for MAOA cDNAs from 2 control males [GM500 and HF53; Hotamisligil and Breakefield, 1991] and from a male affected with mild retardation and impulsive aggressive behavior [AX; Brunner et al., 1993b]. Differences are indicated relative to the cDNA sequence in Hsu et al. [1988]. All sequence variations, with the exception of the introduction of the stop codon in strain AX, appear to be normal polymorphisms which either do not change the deduced amino acid sequence or cause a neutral change in it. Two polymorphisms, T→G at 941, which affects an *Fnu4HI* site [Hotamisligil and Breakefield, 1991], and C→T at 1460, which affects an *EcoR*V site [Ozelius et al., 1988], were noted as differences in the original cDNA clones [Hsu et al., 1988; Bach et al., 1988]. Other variations include A→C at 435, arg→arg (seen in 2/12 cDNAs analyzed to date), A→T at 1076, pro→pro (in 1/12 cDNAs), and A→G at 1609, lys→arg (in 1/12 cDNAs).

In order to expand our search for mutational changes in the *MAOA* gene and to allow resolution of these changes at the genomic level, an additional intronic sequence was obtained by sequencing out from the exons using plasmids containing portions of the human *MAOA* gene, kindly provided by Dr. Zheng Yi Chen (Mass. General Hospital). Sequencing of intron 13 revealed it to contain 203 nucleotides, rather than the approximately 40–50 nucleotides previously estimated [Chen et al., 1991; Grimsby et al., 1991]. For all but two exons, exons 4 and 7, we were able to design flanking intronic primers for PCR that could be used to amplify the entire exon sequences in fragments of <300 kb, suitable for SSCP analysis (Table II). For exon 4, the 5′ primer overlaps 9 bp of exonic sequence, and the 3′ primer overlaps 9 bp of exonic sequence. For exon 7, the 5′ primer is intronic, but the 3′ primer overlaps 9 bp of the exon. In a screen of genomic DNA from 32 additional male controls and 11 Lesch-Nyhan patients, for whom MAO activity measurements were available on fibroblast strains, only a few SSCPs were observed (see Fig. 1). Variations were observed in 5 of the 15 exons. All variant fragments were sequenced across the exons and into at least 3 base pairs of the adjacent intronic sequence on either side of the exon (except in the cases of exons 4 and 7 (see above), and exons 1 and 15, which contained 5′ and 3′ flanking sequences, respectively). No additional nucleotide variations, beyond the polymorphisms noted in Table I, were found in coding sequences. Relative frequencies of coding sequence variations noted in Table I among genomic alleles were 18/57 (including 14 male Parkinson samples [Hotamisligil et al., 1994] for C→T in exon 14; 2/55 for A→C in exon 4; 1/37 for A→G in exon 15; and 1/39 for A→T in exon 9. (The C→T in exon 14 did not give an SSCP shift under the conditions used.) Other SSCP shifts were apparently due to polymorphisms in amplified portions of intronic sequences, none of which would affect splice junctions.

DISCUSSION

MAOA coding sequences appear to be remarkably conserved in a screen of over 40 males for whom activity levels, measured in skin fibroblasts, varied over 100-fold. This conservation is surprising in two regards. First, even complete loss of MAO-A activity is not incompatible with human life. Males with a selective disruption of the *MAOA* gene are not physically disabled, have an apparently normal life span, and are able to reproduce, although some may have low-normal IQs and poor impulse control [Brunner et al., 1993a,b]. Secondly, MAO, and a number of other degradative enzymes, occur as two isozymes with overlapping tissue distribution and substrate specificity. MAO-A and MAO-B can thus "cover" for each other to some extent. Furthermore, there are a number of other enzymes that can degrade the same substrates, e.g., amine oxidase, catechol-O-methyltransferase, and phenol sulfotransferase [see Nagatsu, 1973]. Therefore, the MAO genes

TABLE I. Variations in the Coding Region of *MAOA* From Fibroblast Strains With Different Levels of Activity

Strain[a]	Activity (pmol/min/mg protein)[b]	Coding sequence variations[c]
AX	<1	936, C→T, gln→STOP (exon 8)
GM409	0.9 + 0.2	–
GM497	0.9 + 0.7	435, A→C, arg→arg (exon 4)
GM2037	1.2 + 0.6	–
GM500	1.4 + 0.3	–
Sal Mat	1.6 ± 0.4	435, A→C, arg→arg (exon 4)
GM498	2.7 + 0.7	–
HF8	17.7 + 4.2	941, T→G, arg→arg (exon 8); 1460, C→T, asp→asp (exon 14)
RE11	23.8 + 3.3	1609, A→G, lys→arg (exon 15)
HF53	26.7 ± 3.9	–
HF52	84.5 + 8.0	1076, A→T, pro→pro (exon 9)
LN Bur	179.2 + 30.9	–

[a] All strains are from control males except AX, from a male with the MAO-A-deficiency syndrome [Brunner et al., 1993b], and Sal Mat, GM498, and LN Bur, from Lesch-Nyhan patients [Costa et al., 1980].
[b] MAO activity measured against tryptamine from Edelstein et al. [1978], except AX, from Brunner et al. [1993b].
[c] Nucleotide positions as in Hsu et al. [1988]; variations for HF53 and AX were determined in previous studies [Hotamisligil and Breakefield, 1991; Brunner et al., 1993b, respectively]. No difference indicated by –.

TABLE II. Primers Used for Exon Amplification

Exon	5′ Primer	Location	3′ Primer	Location	Fragment size
1	AGTTGATAGAAGGGTCCTTC	(-)5 to 15	CAGGCCACTGCTACGGTCCACAC	146+1 to 146+27	178
2	CATTTGAGTGTTACGTTG	147-40 to 147-23	TGTAGTTTGAGTGAGATA	241+33 to 241+50	185
3	GGAACCAATTTTTCTCTTTT	242-25 to 242-6	TCACTTGGGTGAAAAGTCAG	379+7 to 379+26	188
4	TATGTTCTAGGGGAAACA	380-10 to 387	ACACATTTACCTCCTTCCC	476 to 484+10	124
5	AGAGGTGGCAGTTACCATCA	485-40 to 485-31	AATTTTGAATGGTCAAGTCT	576+7 to 576+26	157
6	ATTGCAACAGAAAAAACTT	577-111 to 577-94	AGAAAGCAAAATCACAGA	718+40 to 718+57	309
7	CTTTCTTACCTACCTCCTC	719-25 to 719-7	ACTGAGTTACCTCATAATG	860 to 868+10	184
8	GACTGCAGCTCACATCTGAGG	869-47 to 869-27	ACCTCCTGTTCAATAATC	1028 +59 to 1028+76	282
9	CCCATTGATTTTTCTCCT	1029-25 to 1029-8	ATGCAGAAGACCCTGTCTAAC	1125+5 to 1125+25	146
10	ACAGCTGTAACCTGATCATTC	1126-41 to 1126-21	AGCAAAATACAAAAGTT	1179+127 to 1179+144	238
11	TTTTTTTTTGGCTCTGTT	1180-25 to 1180-7	TGCTTTGCTTTACTACTT	1237+51 to 1237+68	150
12	TTTGTTAAAGCAACTATA	1238-82 to 1238-65	AATTTGCACTGAACTCTG	1335+58 to 1335+76	255
13	AGTCATACGGGTGTTTTTT	1336-130 to 1336-113	CCTTCCCGAGACCATTTA	1452 to 1469	467
14	GAAAGCCCAGGCTCTCTC	1448-44 to 1448-27	ATAGTGCCCAGAGTCACCAA	1510+7 to 1510+26	132
15	GACGTTCCAGAGGTAGAAAT	1511 to 1530	ACATGAGTGATCTACACTG	1862 to 1880	369
*13	ATTTACCCTGCCCACCTT	1336-56 to1336-38	CTGGGCTTTCCTGCTTAC	1401 to 1401+18	185

* Denotes new primers used for SSCP to shorten intronic sepuences.

should be able to sustain mutations in the coding sequence that affect levels of activity or substrate affinities, as do genes for other degradative enzymes, e.g., alcohol dehydrogenase [Crabb et al., 1989], N-acetyltransferase [Deguchi et al., 1990], and debrisoquine hydroxylase [Smith et al., 1992]. It is also possible that some mutations in the MAO-A gene in these samples were missed, as SSCP analysis was only carried out under one set of electrophoretic conditions, and 5 of the 15 fragments analyzed were >250 bp in size, or above the ideal limit for SSCP analysis. In only 12 cases have the coding regions of the cDNAs been sequenced in their entirety. A number of other methods are available for detecting mutations in such PCR fragments, including enzyme mismatch cleavage and denaturing gradient gel electrophoresis [reviewed in Dean, 1995].

Hotamisligil and Breakefield [1991] have indicated that the *MAOA* locus itself is a strong determinant of levels of enzyme activity as measured in cultured skin fibroblasts. This conclusion was based on a highly significant association of specific alleles, marked by three normal polymorphisms in the gene, and by low and high levels of MAO activity in control males [Hotamisligil and Breakefield, 1991; Breakefield et al., 1994]. In addition, we found a significantly different distribution of *MAOA* alleles between Parkinson patients and controls [Hotamisligil et al., 1994]; although this was not noted in a similar study in which the MAOA alleles were marked by a single polymorphism [Kurth et al., 1993]. In the present study we did not find a difference in the coding sequence of the *MAOA* gene which could account for differences in levels of activity. It is possible that these activity differences are influenced by sequence variations in regulatory regions, which determine rates of transcription; in intronic regions, which affect splicing efficiency; or in untranslated regions of the message which affect transport, processing, or stability of the message. These types of changes may be difficult to elucidate due to the high degree of other, apparently inconsequential, variations found in such noncoding sequences.

This study identifies a number of new polymorphisms in the *MAOA* gene which will help in following its inheritance in families and in allele association studies. More importantly, primers are provided which allow PCR amplification of all 15 exons of the *MAOA* gene in genomic DNA in fragments of <300 bp, convenient for mutational analysis and sequencing. These primer sets will be critical in screening genomic DNA to determine the frequency of mutations in the *MAOA* gene and their phenotypic manifestations in other individuals. Although the spectrum of symptoms is not known at this time, it is safe to assume that, as with people taking MAO inhibitors for depression or hypertension, MAO-A-deficient males would be susceptible to hypertensive crises in response to dietary intake of certain amines, such as tyramine and tryptamine [Murphy, 1977; Lavine et al., 1993]. The present study supports the premise that disruptive mutations in the MAOA gene are not common in the population.

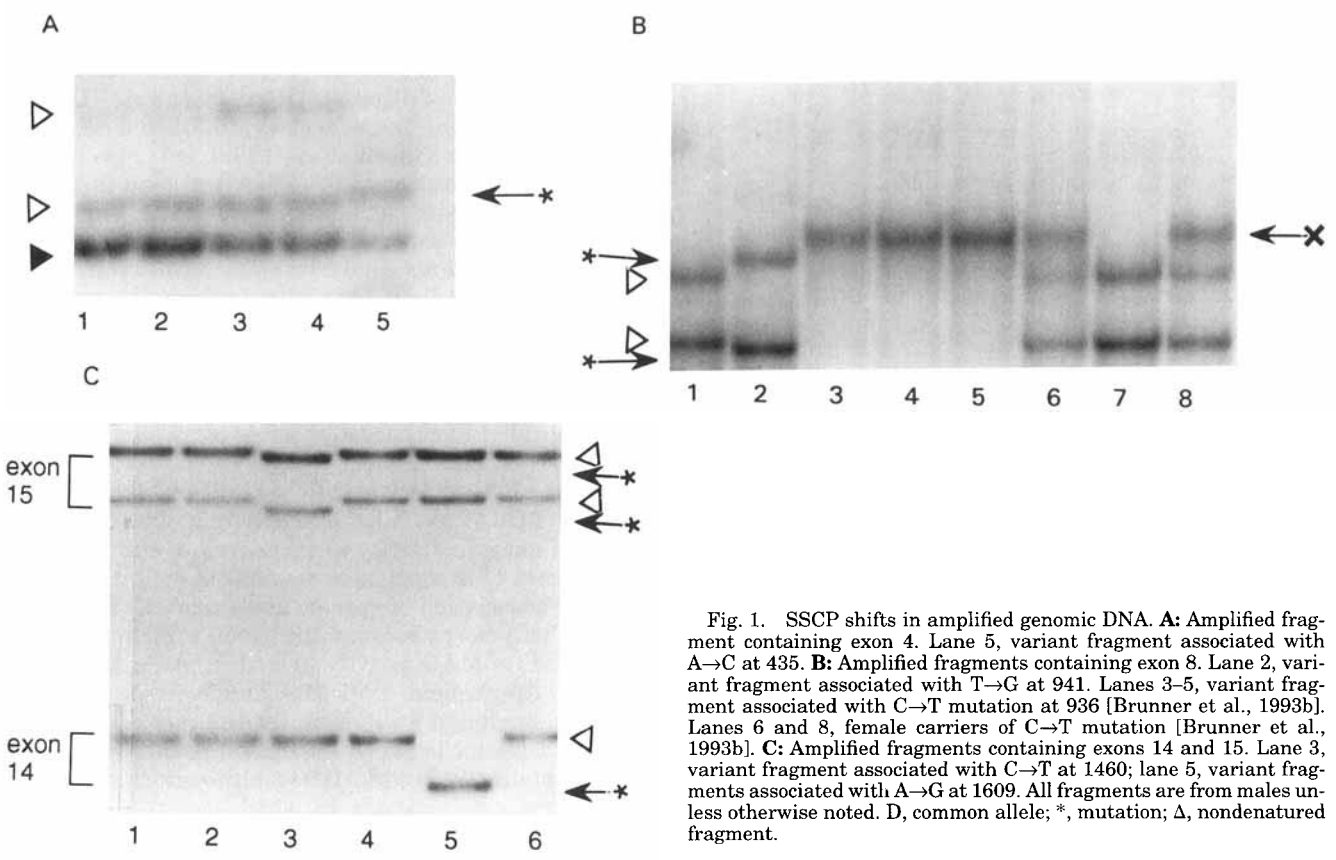

Fig. 1. SSCP shifts in amplified genomic DNA. **A:** Amplified fragment containing exon 4. Lane 5, variant fragment associated with A→C at 435. **B:** Amplified fragments containing exon 8. Lane 2, variant fragment associated with T→G at 941. Lanes 3–5, variant fragment associated with C→T mutation at 936 [Brunner et al., 1993b]. Lanes 6 and 8, female carriers of C→T mutation [Brunner et al., 1993b]. **C:** Amplified fragments containing exons 14 and 15. Lane 3, variant fragment associated with C→T at 1460; lane 5, variant fragments associated with A→G at 1609. All fragments are from males unless otherwise noted. D, common allele; *, mutation; Δ, nondenatured fragment.

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